ELSEVIER

FEMS Microbiology Letters 215 (2002) 163–168

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Microbial survival of space vacuum and extreme ultraviolet irradiation: strain isolation and analysis during a rocket flight

Roya Saffary [a], Renu Nandakumar [a], Dennis Spencer [b], Frank T. Robb [b], Joseph M. Davila [c], Marvin Swartz [c], Leon Ofman [c,d], Roger J. Thomas [c], Jocelyne DiRuggiero [a,b,*]

[a] *Department of Cell Biology and Molecular Genetics, University of Maryland, 3221 H.J. Patterson Hall, College Park, MD 20742, USA*
[b] *Center of Marine Biotechnology, University of Maryland Biotechnology Institute, Baltimore, MD USA*
[c] *Laboratory for Astronomy and Solar Physics, NASA Goddard Space Flight Center, Greenbelt, MD USA*
[d] *Catholic University of America, Washington, DC, USA*

Received 14 May 2002; received in revised form 12 August 2002; accepted 16 August 2002

First published online 12 September 2002

Abstract

We have recovered new isolates from hot springs, in Yellowstone National Park and the Kamchatka Peninsula, after γ-irradiation and exposure to high vacuum (10^{-6} Pa) of the water and sediment samples. The resistance to desiccation and ionizing radiation of one of the isolates, *Bacillus* sp. strain PS3D, was compared to that of the mesophilic bacterium, *Deinococcus radiodurans*, a species well known for its extraordinary resistance to desiccation and high doses of ionizing radiation. Survival of these two microorganisms was determined in real and simulated space conditions, including exposure to extreme UV radiation (10–100 nm) during a rocket flight. We found that up to 15 days of desiccation alone had little effect on the viability of either bacterium. In contrast, exposure to space vacuum ($\sim 10^{-6}$ Pa) decreased cell survival by two and four orders of magnitude for *Bacillus* sp. strain PS3D and *D. radiodurans*, respectively. Simultaneous exposure to space vacuum and extreme UV radiation further decreased the survival of both organisms, compared to unirradiated controls. This is the first report on the isolated effect of extreme UV at 30 nm on cell survival. Extreme UV can only be transmitted through high vacuum, therefore its penetration into the cells may only be superficial, suggesting that in contrast to near UV, membrane proteins rather than DNA were damaged by the radiation.

Keywords: Extremophile; Space vacuum; Extreme ultraviolet; Space survival; Ionizing radiation

1. Introduction

Microorganisms on our planet display tremendous diversity, some colonizing what we might consider extreme environments such as deep-sea hydrothermal vents, hypersaline lakes, ice-covered Antarctic lakes, the interiors of rocks and deep subsurface aquifers [1,2]. By studying microbial communities that are adapted to extreme conditions, and establishing the limits to life on our own planet, we may get insights into the potential of other worlds to support life, and thus determine whether life as we know it could exist elsewhere in the galaxy. These studies may be relevant to questions concerning inadvertent transmission of terrestrial extremophiles to moons and planets during interplanetary space flights, and the extraterrestrial origin of life as described in the so-called Panspermia hypothesis. This hypothesis was first proposed by Arrhenius early in the last century and suggests that terrestrial life might have originated elsewhere in the galaxy and have been transmitted to our planet through space [3].

Thermophiles have recently been described that can grow up to 113°C, which is the upper temperature for life as we know it [4]. We chose thermophilic microorganisms for our studies based on their resistance to extreme temperature, and because we have found that hyperthermophiles, such as *Pyrococcus* spp., are highly resistant to ionizing radiation [5–7]. This is significant since high energy ionizing radiation typical of space conditions is known to be highly lethal to microorganisms [8]. Thermo-

* Corresponding author. Tel.: +1 (301) 405-4598; Fax: +1 (301) 314-9081.
E-mail address: jd92@umail.umd.edu (J. DiRuggiero).

0378-1097/02/$22.00
PII: S0378-1097(02)00953-9

philes have robust macromolecules and effective DNA repair systems [9,10], and they inhabit environments such as deep-sea hydrothermal vents that some argue might resemble subsurface locations on Mars and Europa [2]. In addition, hydrothermal deep-sea vents have been proposed as possible sites for the origin of life on Earth, and this notion could be extended to other planets if one believes that life evolved somewhere else in space and was then transported to Earth [3].

Several studies have investigated the effects of space vacuum and UV irradiation on the survival of microorganisms using mainly bacterial spores [11], and most recently osmophilic microorganisms [12]. These studies revealed that *Bacillus* spores survived nearly 6 years in space when shielded from UV radiation, whereas when exposed to the full range of space conditions, only a small proportion of the spores from the innermost part of the samples were able to survive [13]. A higher survival rate than that of *Bacillus* spores was reported for two halophiles, *Synechococcus* sp. and *Haloarcula* sp., when exposed to both UV (whole spectrum) and space vacuum [12]. Both organisms inhabit high salt environments, suggesting that, under anhydrobiosis, salt crystals might act as 'sunscreens' against the deleterious effects of UV radiation [12]. The killing effect and damage to the DNA caused by UV radiation from 200 to 400 nm, and especially at 254 nm, have been extensively studied [14]. In contrast, very little is known about the specific effects on cells of extreme UV (EUV) at wavelengths from 10 to 100 nm.

EUV can only be transmitted through high vacuum, and as a consequence the damage it inflicts on cells may be rather different than that produced by exposure to UV from 200 to 400 nm. In this study we examined the effects of space vacuum and EUV, singly and in combination, on the survival of vegetative cells from two microorganisms, *Deinococcus radiodurans* and an isolate from a hot spring in Yellowstone National Park.

2. Materials and methods

2.1. Growth conditions, sampling, strain isolation

Cells were grown on TGY broth (5 g tryptone, 3 g yeast extract, 1 g glucose per liter) with shaking at 220 rpm (Innova 4080, New Brunswick Scientific, Edison, NJ, USA) or plated on TGY broth solidified with 15 g l^{-1} of agar. Incubation varied from 65 to 75°C. Water and sediment samples were collected from hot springs in Yellowstone National Park and the Kamchatka Peninsula in Russia, and exposed to either γ-irradiation at a dose of 3500 Gy, or high vacuum at 10^{-6} Pa for 3 days. Following exposure to the challenges, samples were used to inoculate liquid culture media. The cultures were incubated at 65, 70 and 75°C for 3–5 days, followed by plating and incubation under the same conditions. *D. radiodurans* R1 was a gift of John Battista (Louisiana State University).

2.2. RFLP and sequencing

Genomic DNA extraction from 13 Yellowstone isolates was performed as described in Ausubel et al. [15]. Polymerase chain reaction (PCR) was used to amplify 16S rRNA genes with the following primers: 5′-AGAGTTTGATCCTGGCTCAG-3′ (*Escherichia coli* position 8–27) and 5′-GGTTACCTTGTTACGACTT-3′ (*E. coli* position 1510–1492). Restriction fragment length polymorphism (RFLP) analysis was carried out by restricting PCR products with *Hha*I and *Hae*III, as specified by the manufacturer (Promega, Madison, WI, USA). Digests were analyzed by electrophoresis in 4% MetaPhor agarose (FMC BioProducts, Rockland, ME, USA). PCR products were cloned using the TOPO TA cloning system (Invitrogen, Carlsbad, CA, USA) and sequenced using BigDye terminator reactions, vector primers, and ABI 3100 automated sequencers (Perkin Elmer, Boston, MA, USA). The *Bacillus* sp. strain PS3D 16S rRNA sequence was submitted to the GenBank/EMBL/DDBJ databases under accession number AF517644. Neighbor-joining analysis was performed using the program NEIGHBOR of the PHYLIP 3.6 package [16].

2.3. Exposure to desiccation, high vacuum and ionizing radiation

Cells grown on TGY broth for 16 h at 70°C were centrifuged at 4°C, 17 000 × *g*, washed once with 10 mM $MgSO_4$, 5% trehalose, and resuspended in the same buffer at a concentration of 10^8 cells ml^{-1}. One-ml aliquots of cell suspension were filtered onto 25-mm-diameter, 0.22-μm polycarbonate filters. Filters were air-dried and either stored in a desiccation jar with Drierite desiccant (desiccated samples), or exposed to high vacuum at the NASA Goddard Space Flight Center (GSFC, Greenbelt, MD, USA) (high vacuum samples). For the latter, filters were placed into sterile Costar 6-well plates (Corning, Acton, MA, USA) with covers perforated by several 3-mm holes to unsure homogeneous vacuum. In each of the vacuum runs, scroll pumps evacuated the chamber to a pressure below 40 μm, vac-sorb pumps brought it below 5 μm, and finally cryopumps were used to maintain a high vacuum of 10^{-6} Pa. The process took 2.5 h. Return to atmospheric pressure was accomplished in 10 min. Following exposure to vacuum, filters were incubated in 5 ml of 10 mM $MgSO_4$, 5% trehalose at 4°C for 2 h with gentle agitation (LabLine Maxi Rotator at a setting of 10, Melrose Park, IL, USA). Dilutions were plated on TGY plates and incubated at 65–75°C for 3–5 days before recording number of colony forming units. Control filters were processed immediately without drying. Three plates minimum were

counted per dilution, and each experiment was performed in triplicate.

Cells exposed to ionizing radiation were irradiated in their culture media with a ^{60}Co γ-ray source at the National Institute for Standards and Technology (Gaithersburg, MD, USA; dose rate: 76 Gy min^{-1}), and cell counts were performed as described above.

2.4. Rocket flight

Exponential cultures of *D. radiodurans* and *Bacillus* sp. strain PD3D were washed with 10 mM $MgSO_4$, 5% trehalose and filtered onto 25-mm-diameter, 0.22-μm polycarbonate filters. Observation of the filters under the microscope confirmed that 10^8 cells per filter resulted in a single layer of cells. Filters were mounted onto specifically designed metal sample holders, with the control filter shielded from EUV exposure by a metal plate. The design of those modules was described elsewhere [17]. Two duplicate modules (filters+holders), one for each microorganism, were transported to White Sands Missile Range, New Mexico, USA, and mounted on the payload of a Terrier Black Brant Rocket carrying the Solar EUV Spectrograph (SERTS). The rocket payload reached an altitude of 304 km in 283.5 s, and remained steadily pointing at the sun for a total of 395 s. An aluminum filter placed between the sun and the sample holders, combined with the focusing mirror's reflectivity response and the solar spectrum, assured that only EUV radiation at 30.4 nm reached the test sample. The irradiation dose received by the microorganisms at 30.4 nm was 6×10^{12} photons cm^{-2}. The temperature of each sample was monitored in real time from pre-launch throughout recovery, and was found to reach a maximum of 40°C. The cells stayed desiccated for a total of 15 days, including 5 days under high vacuum while the SERTS payload was evacuated after being mated to the rocket motors and awaiting launch. Within 2 h of launch, the payload was recovered. The cell holders were placed into sterilized bags and returned to the laboratory, where viability of the cells was measured as described above. Control filters of dehydrated cells remain stored in desiccation jars with Drierite desiccant during the length of the experiment.

3. Results and discussion

3.1. Resistance of isolates to desiccation, high vacuum and γ-irradiation

Samples from Yellowstone National Park and the Kamchatka Peninsula were exposed to high vacuum and radiation to specifically select for resistant strains. Under those conditions, we isolated 13 strains with optimum growth temperatures of 70°C, nine from high vacuum and four from radiation exposure of the samples.

Survival of the 13 isolates was determined following γ-irradiation at doses up to 5500 Gy and exposure to desiccation and to high vacuum (10^{-6} Pa) for 3 days. Desiccation of the cells decreased survival by an average of 50%, whereas only 10% of the cells survived following exposure to high vacuum (Fig. 1). Cells isolated with high vacuum as selective factor showed the same resistance to high vacuum (Fig. 1B) as cells isolated following γ-irradiation (Fig. 1A). We found that all the strains were highly resistant to ionizing radiation with D_{37} (dose for 37% survival) above 3500 Gy (Fig. 2). In comparison, the D_{37} for *E. coli* is lower than 100 Gy. We observed up to 7% survival at the very high dose of 5500 Gy, in strains isolated following ionizing radiation (Fig. 2A). In contrast, the strains isolated with high vacuum as selective factor showed less than 1% survival following exposure to 5500 Gy (Fig. 2B). Although both ionizing radiation and desiccation induce DNA double-strand breaks [14,18], these results suggested that exposure to γ-irradiation produces additional cellular damage that is not repaired as efficiently by the strains isolated with high vacuum as selective factor.

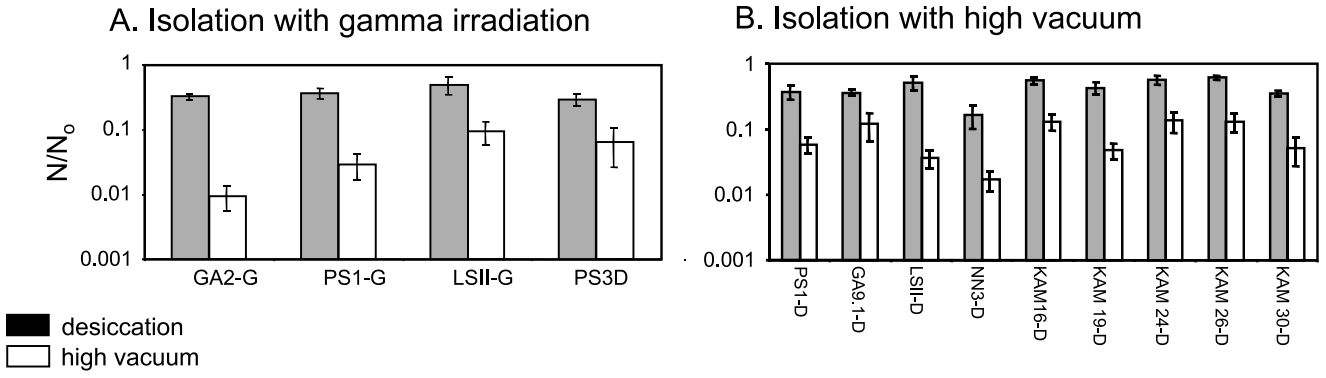

Fig. 1. Cell survival following exposure to high vacuum of 13 strains isolated (A) with ionizing radiation as selective factor and (B) with high vacuum as selective factor. N = number of viable cells in the challenged sample; N_o = number of viable cells in the control. Error bars represent standard deviation for triplicate experiments.

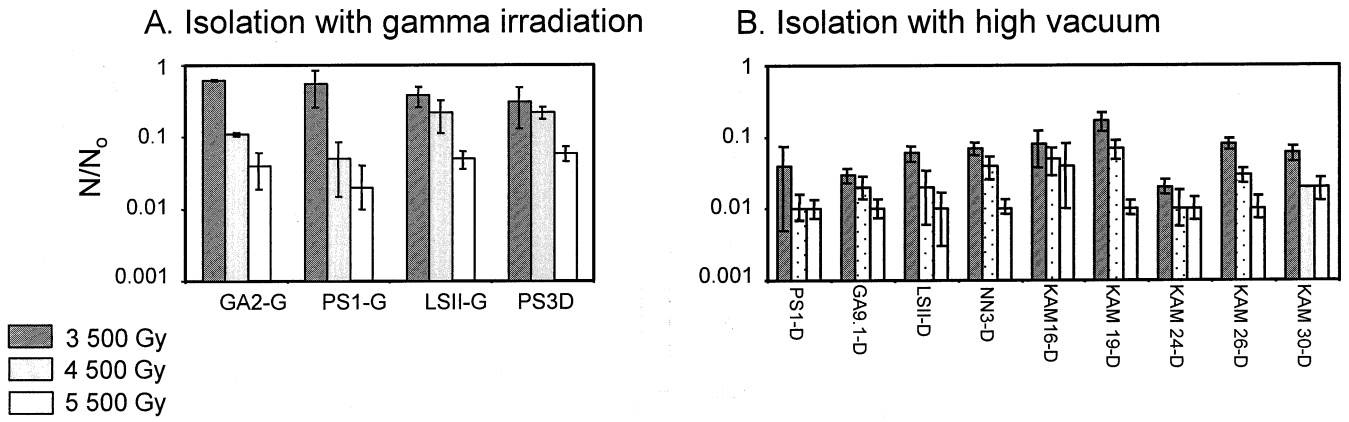

Fig. 2. Cell survival following exposure to ionizing radiation of 13 strains isolated (A) with ionizing radiation as selective factor and (B) with high vacuum as selective factor. N = number of viable cells in the challenged sample; N_o = number of viable cells in the control.

3.2. Molecular analysis of the isolated strains

Using oligonucleotides with highly conserved sequences as primers, we amplified the 16S rRNA of the 13 isolates by PCR. The PCR products were subjected to restriction digest with two enzymes, *Hae*III and *Hha*I, and the DNA was visualized by agarose gel electrophoresis. All the strains displayed an identical pattern of six and five bands for *Hae*III and *Hha*I, respectively (data not shown), indicating that they were all very closely related. This was unexpected since we collected samples from widely separate locations, Yellowstone National Park and the Kamchatka Peninsula in eastern Russia. These results suggest that the distribution of microbial strains highly resistant to desiccation, ionizing radiation and high temperature is not ubiquitous, but rather defined by environmental factors. It is tempting to speculate that strains with these physiological properties are distributed by atmospheric circulation, as a consequence of their ability to survive prolonged exposure in the stratosphere.

We sequenced the 16S rRNA for one of the strain, PS3D, and constructed a phylogenetic tree (Fig. 3). The PS3D sequence clustered with thermophilic members of the *Bacillus* genus, and is more closely related to *Bacillus stearothermophilus*. The two 16S rRNA sequences differ by two nucleotides out of 1440 analyzed, suggesting that PS3D might be a new species of *Bacillus*. Although the isolates we used in this study were *Bacillus*, we did not observe any spores during exposure to challenges, upon return from the rocket flight or during manipulation of the cultures, suggesting that the vegetative cells themselves are highly resistant to ionizing radiation and high vacuum.

3.3. Exposure of microorganisms to space vacuum and EUV during a rocket flight

We have determined the survival of thermophilic microorganisms exposed to space conditions and EUV radiation during a rocket flight. Two microorganisms, *D. radiodurans* and *Bacillus* sp. strain PD3D (isolated from Yellowstone National Park, optimum growth temperature 70°C), were selected for their resistance to desiccation and high vacuum. For both microorganisms desiccation had little effect on the cell survival compared to the non-desiccated control (Fig. 4). Exposure to space vacuum ($\sim 10^{-6}$ Pa), however, decreased cell survival by two and four orders of magnitude for *Bacillus* sp. strain PD3D and *D. radiodurans* respectively. The most interesting result of this experiment is that exposure to EUV radiation decreased the survival of both organisms by an additional order of mag-

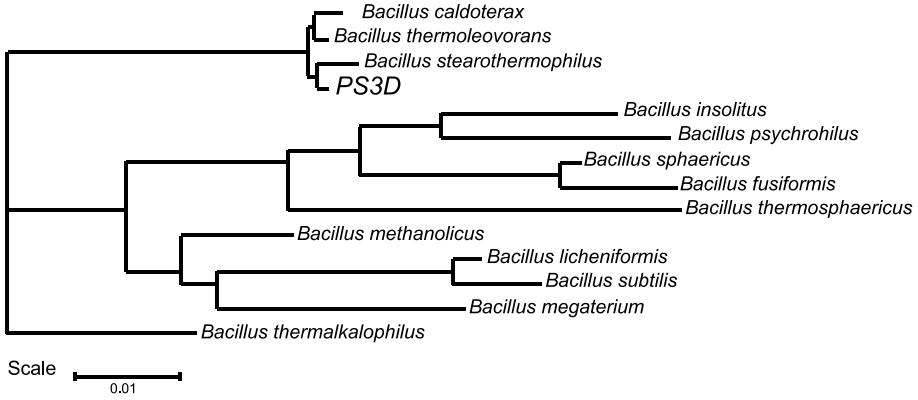

Fig. 3. Neighbor-joining phylogenetic tree constructed with the Neighbor program of the PHYLIP 3.6 package. Scale bar corresponds to 0.01 substitution per position.

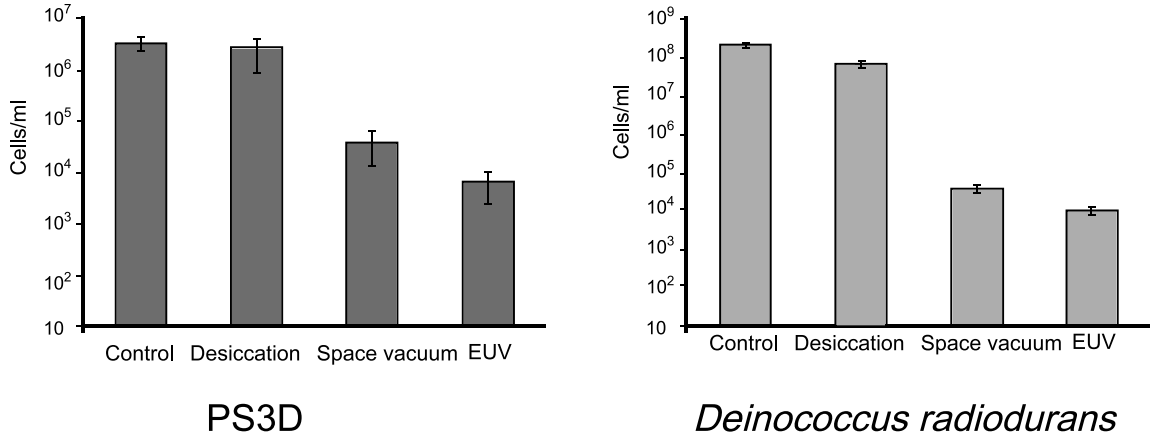

Fig. 4. Cell survival following the rocket flight for the Yellowstone National Park isolate, *Bacillus* sp. strain PS3D, and *D. radiodurans*.

nitude. This is the first measurement of the effect of isolated EUV on cell survival.

D. radiodurans cells were exposed to high vacuum in the GSFC vacuum chamber for the same length of time as cells exposed to vacuum during the rocket flight. The results, shown in Fig. 5, indicate a higher loss of viability for cells exposed to space vacuum when compared to those exposed to high vacuum. In both cases, the vacuum level was measured at 10^{-6} Pa, however, the space vacuum can be considered a composite of stress factors, including zero gravity and ionizing radiation. Although we do not have a specific hypothesis to explain the difference in survival between space and ground, these results emphasize the necessity for space experiments in addition to those performed on the ground.

These results provide the basis for further studies of the effects of space vacuum and EUV on desiccation-resistant microorganisms. It is also a proof of the utility of using rocket flights as preliminary experiments to design longer duration space experiments that could be done, for example, during Space Shuttle flights or in the International Space Station. We established that a vacuum of 10^{-6} Pa decreased the survival of desiccation-resistant microorganisms, and that exposure to space vacuum caused a greater decline in survival when compared to ground vacuum. The reduction of survival in cells exposed to EUV is the most significant result in this study. EUV can only be transmitted through high vacuum and one can surmise that its penetration into the cells is limited. We suggest that damage to the cell membrane and its proteins may be responsible for the loss of cell viability when cells in high anhydrobiosis are exposed to EUV. In ground-based experiments, one could use EUV radiation produced by a synchrotron to expose cells to higher doses in order to determine the types of cellular lesions resulting from EUV exposure. These experiments will require the design and construction of specific equipment, using thin metal filters of various materials to deliver several narrow EUV wavelength bands.

Acknowledgements

This work was supported by Grant NCC8-175/NCC9-147 from the NASA micro-gravity program. We thank Rhonda Holley-Shanks for her technical support and all the people at White Sands Missile Range who made our participation in the SERTS flight possible. The SERTS

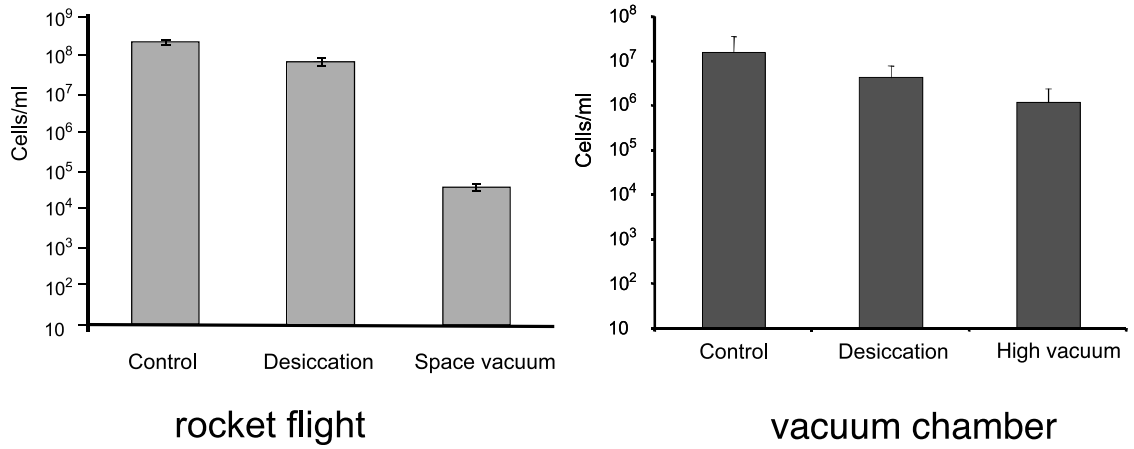

Fig. 5. Cell survival following exposure to high vacuum during the rocket flight and in the desiccation chamber at NASA GSFC for *D. radiodurans*.

rocket program was separately funded under NASARTOP Grant 879-11-38. F.T.R. is funded by NSF Grant MCB-9809352.

References

[1] Rothschild, L.J. and Mancinelli, R.L. (2001) Life in extreme environments. Nature 409, 1092–1101.
[2] Horneck, G. (2000) The microbial world and the case for Mars. Planet. Space Sci. 48, 1053–1063.
[3] Line, M.A. (2002) The enigma of the origin of life and its timing. Microbiology 148, 21–27.
[4] Blochl, E., Rachel, R., Burgraf, S., Hafenbradl, D., Jannasch, H.W. and Stetter, K.O. (1997) *Pyrolobus fumarii*, gen. and sp. nov., represents a novel group of archaea, extending the upper limit temperature for life to 113°C. Extremophiles 1, 14–21.
[5] DiRuggiero, J., Santangelo, N., Nackerdien, Z., Ravel, J. and Robb, F.T. (1997) Repair of extensive ionizing-radiation DNA damage at 95°C in the hyperthermophilic archaeon *Pyrococcus furiosus*. J. Bacteriol. 179, 4643–4645.
[6] Gerard, E., Jolivet, E., Prieur, D. and Forterre, P. (2001) DNA protection mechanisms are not involved in the radioresistance of the hyperthermophilic archaea *Pyrococcus abyssi* and *P. furiosus*. Mol. Genet. Genomics 266, 72–78.
[7] Kopylov, V.M., Bonch-Osmolovskaya, E.A., Svetlichnyi, V.A., Miroshnichenko, M.L. and Skobkin, V.S. (1993) γ-irradiation resistance and UV-sensitivity of extremely thermophilic archebacteria and eubacteria. Mikrobiologiya 62, 90–95.
[8] Horneck, G., Bucker, H. and Reitz, G. (1994) Long term survival of bacterial spores in space. Adv. Space Res. 14, 41–45.
[9] DiRuggiero, J., Brown, J.R., Bogert, A.P. and Robb, F.T. (1999) DNA repair systems in Archaea: Mementos from the last universal common ancestor? J. Mol. Evol. 49, 474–484.
[10] Grogan, D.W. (2000) The question of DNA repair in hyperthermophilic archaea. Trends Microbiol. 8, 180–185.
[11] Nicholson, W.L., Munakata, N., Horneck, G., Melosh, H.J. and Setlow, P. (2000) Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. Microbiol. Mol. Biol. Rev. 64, 548–572.
[12] Mancinelli, R.L., White, M.R. and Rothschild, L.J. (1998) Biopan survival I: exposure of the osmophiles *Synechococcus* sp. (Nageli) and *Haloarcula* sp. to the space environment. Adv. Space Res. 22, 327–334.
[13] Horneck, G., Bucker, H. and Reitz, G. (1994) Long-term survival of bacterial spores in space. Adv. Space Res. 14, 41–45.
[14] Friedberg, E.C., Walker, G.C. and Siede, W. (1995) DNA Repair and Mutagenesis. ASM Press, Washington, DC.
[15] Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Smith, J.A., Seidman, J.G. and Struhl, K. (1987) Current Protocols in Molecular Biology. Greene Publishing Associates and Wiley-Interscience, New York.
[16] Felsenstein, J. (1993) PHYLIP (Phylogeny Inference Package) version 3.57c. Distributed by the author. Department of Genetics, University of Washington, Seattle, WA.
[17] DiRuggiero, J., Nandakumar, R., Eisen, J.A., Schwartz, M., Thomas, R., Davila, J., Ofman, L. and Robb, F.T. (2002) Genomic and physiological studies on extremophiles: model systems for exobiology. In: Pavlov, A. (Ed.), Frontiers of Astrobiology. Proceedings of the Astrobiology Workshop, St. Petersburg, Russia (in press).
[18] Battista, J.R. (2000) Radiation resistance: the fragments that remain. Curr. Biol. 10, 204–205.